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Original Paper

WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer

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Abstract

To detect novel Wnt-pathway genes involved in tumourigenesis, this study analysed the RNA expression levels of 40 genes of the Wnt pathway by chip hybridization of microdissected matched pairs of 54 primary prostate carcinomas. Eleven genes showed greater than two-fold differential expression in at least 10% of prostate cancers. Three of these genes encode extracellular components of the Wnt pathway (WNT2, WIF1, SFRP4); two are receptors (FZD4, FZD6); two belong to the intracellular signal cascade (DVL1, PPP2CB); one regulates transcription (TCF4); and three represent genes regulated by this pathway (CCND2, CD44, MYC). While SFRP4, FZD4, FZD6, DVL1, TCF4, and MYC are up-regulated, WIF1, WNT2, PPP2CB, CCND2, and CD44 are down-regulated in certain prostate cancer patients. Wnt inhibitory factor 1 (WIF1) and secreted frizzled related protein (SFRP4) showed the most significant aberrant expression at the RNA level. WIF1 was down-regulated in 64% of primary prostate cancers, while SFRP4 was up-regulated in 81% of the patients. Immunohistochemical analysis using a polyclonal antibody revealed strong cytoplasmic perinuclear WIF1 expression in normal epithelial cells of the prostate, breast, lung, and urinary bladder. Strong reduction of WIF1 protein expression was found in 23% of prostate carcinomas, but also in 60% of breast, 75% of non-small cell lung (NSCLC), and 26% of bladder cancers analysed. No significant association between WIF1 down-regulation and tumour stage or grade was observed for prostate, breast or non-small cell lung carcinomas, indicating that loss of WIF1 expression may be an early event in tumourigenesis in these tissues. However, down-regulation of WIF1 correlated with higher tumour stage in urinary bladder tumours (pTa versus pT1–pT4; $p = 0.038$). Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: WIF1; immunohistochemistry; prostate; bladder; breast; lung; cancer

Introduction

Gene expression analysis using DNA microarray technology is a useful tool for detecting new genes that may be implicated in the development of cancer or that represent novel marker molecules. Recently, profiling techniques have been shown to be especially powerful in detecting single markers and RNA signatures that have the potential to supplement and improve cancer diagnosis and prognosis [1–4]. Especially promising is the possibility of simultaneous evaluation of all participants of a particular regulatory pathway frequently altered in cancer.

To gain new insights into the gene expression profiles of medically important human cancers, to screen for cancer-related candidate genes, and to study the RNA expression pattern of important signal transduction pathways, we have designed a proprietary cancer microarray based on Affymetrix technology containing 3000 genes. About 50% of these genes were nominated based on their differential expression as shown by bioinformatic analysis of tumour-derived EST libraries [5]. The other half represent known tumour-associated candidate genes including most members of key signal transduction pathways such as the Wnt pathway [6–9] (Table 1).

Binding of Wnt proteins to the frizzled receptors activates the intracellular Dishevelled, which inhibits GSK3β and allows the cytoplasmic accumulation of stabilized β-catenin. β-catenin then enters the

Table 1. Expression of genes of the Wnt pathway in prostate cancer. Expression was analysed in 108 corresponding normal (N) and tumour (T) prostate samples (54 patients) by chip hybridization. For each tissue pair, the fold change ($F = T/N$) of the expression values was calculated. Fold changes of $F > 2$ and $F < 0.5$ were regarded as over- and under-expression, respectively

Genes	Expressed in	Over-expressed	Under-expressed	Ratio of over-/under-expression
Extracellular				
SFRP1	53	7	8	
SFRP3	44	10	11	
SFRP4	37	30	7	4.3
SFRP5	0	—	—	
WIF1	42	2	27	<0.1
WNT1	18	9	9	
WNT2	43	4	13	0.3
WNT2B	44	9	12	
WNT5A	36	8	9	
WNT7A	0	—	—	
WNT8B	0	—	—	
WNT10B	4	4	0	
WNT14	0	—	—	
Signal cascade				
APC	1	0	1	
AXIN	8	6	2	
CTNNB1	54	2	1	
DVL1	50	6	0	>6
DVL2	1	1	0	
DVL3	0	—	—	
FZD2	0	—	—	
FZD3	13	11	7	
FZD4	53	8	2	4
FZD5	0	—	—	
FZD6	32	12	5	2.4
FZD9	0	—	—	
GSK3B	16	13	3	
PPP2CB	54	2	5	0.4
PPP2R1B	0	—	—	
PPP2R5A	52	4	0	
WISP2	3	0	4	
Nuclear				
CREBP	52	0	0	
TCF4	52	5	1	5
TCF6L1	52	3	1	
TCF7L2	0	—	—	
Target genes				
CCND1	53	1	4	
CCND2	54	2	10	0.2
CCND3	50	2	1	
CD44	50	2	10	0.2
JUN	32	9	14	
MMP7	44	18	12	
MYC	46	24	3	8

nucleus, activates transcription factors, and leads to the transcription of target genes such as cyclin D, c-myc, or CD44. In the absence of a Wnt signal, β-catenin is phosphorylated by GSK3β and thus labelled for degradation by the covalent addition of ubiquitin [10].

Regulation of signal transduction of the Wnt pathway may occur at different levels, including extracellular components of the signalling cascade [11–13]. In humans, the Wnt10b gene is up-regulated in a small subset of human breast carcinomas, the Wnt2 gene is overexpressed in human colorectal carcinomas, and the Wnt5a gene in malignant melanoma [14–16]. In order to initiate signal transduction, the Wnt protein binds to the cysteine-rich domain (CRD) of the transmembrane frizzled receptor protein. The binding of Wnt ligands to the frizzled receptor can be reduced or even prevented by competitive binding of secreted frizzled related proteins (SFRP) to the Wnt ligands, thus preventing signalling through the frizzled receptor. The final effect of this extracellular signal modulation is a decrease in the intracellular level of β-catenin [17,18]. Recent studies have suggested the involvement of SFRP1 gene expression in breast cancer [19–22]. In addition, loss of expression of SFRP1, SFRP2, SFRP4, and SFRP5 has recently been described as

due to promoter hypermethylation in colon and gastric cancer [23].

There are several other proteins including WIF1, Dickkopf, and Cerberus that also have the ability to bind Wnt proteins and thus compete with the binding of Wnt proteins to the frizzled receptor. These proteins are structurally diverse and mainly function as secreted inhibitors of Wnt signalling [10]. WIF1 is an evolutionarily conserved protein containing five EGF-like domains and one WIF domain, which mediates the Wnt binding of WIF1 [24]. Overexpression of WIF1 in *Xenopus* embryos blocks the Wnt8 pathway and results in a phenotype, as seen by overexpression of the Wnt antagonist SFRP-3. These *in vivo* results show the ability of Wnt proteins to bind structurally diverse extracellular antagonists.

Using custom arrays, we have studied the RNA expression of most components of the Wnt signal transduction pathway in prostate cancer by using microdissected samples of both tumour and normal tissue samples. We show that 11 genes of the Wnt pathway are differentially expressed at the RNA level in prostate cancer. Most prominently, WIF1 is down-regulated in 64% of the tumours and SFRP4 is up-regulated in 81% of all cases. We demonstrate that WIF1 is also down-regulated at the protein level in prostate cancer and a number of other major solid tumours including breast, NSCLC, and bladder cancers. These data suggest that WIF1 is a potential tumour suppressor and may be required to compete for Wnt ligands. Loss of WIF1 in tumours may lead to the unrestricted binding of Wnt ligands to the frizzled receptor, followed by enhanced transcription of target genes of the Wnt pathway.

Materials and methods

Patient characteristics and tumour specimens

The expression profiles of 54 prostate cancer patients (age 47–73 years; Gleason score 4–9; tumour stage pT2a–pT4; pre-operative PSA 2–30 ng/ml) were analysed by DNA chip hybridization and quantitative RT-PCR. Included in this study were prostate cancer patients who underwent radical prostatectomy at the Department of Urology at the University Hospital Charité from 1998 to 2000.

The specimens were sectioned by a pathologist according to diagnostic standards. Selected tissue slices were snap-frozen in liquid nitrogen and stored until further processing at −80 °C. For immunohistochemical analysis, formalin-fixed and paraffin-embedded prostate, breast, lung, and bladder cancer specimens were obtained from the Institute of Pathology, University of Regensburg, Germany. Histological diagnosis, tumour stage, and grade were reviewed by one pathologist (AH) according to the WHO and the UICC classification of tumours.

Informed consent was obtained from all patients and the study was approved by the Institutional Review Board. An overview of the histopathological characteristics of the tissue samples stained using the WIF1 antibody is given in Table 2.

Microdissection, RNA preparation, and array hybridization

Microdissection was performed as described previously [25]. In brief, 5–20 5-µm frozen sections were obtained for each case and stored at −80 °C. The slides were immediately fixed in 80% ethanol and stained with methylene blue for approximately 15 s. The tumour was separated from stromal cells by microdissection with a needle (22 G) under an inverted microscope (40 × magnification) or by using laser microdissection (PALM, Wolfrathshausen, Germany). All samples contained at least 90% tumour cells. After microdissection, cells were collected in GTC buffer containing 2% β-mercaptoethanol for further RNA preparation. Poly-A^+-RNA was prepared using

Table 2. WIF1 immunohistochemistry of a total 162 matched pairs of tumour and normal tissues from bladder, prostate, breast, and lung cancer patients. Histopathological diagnoses were reviewed according to the WHO classification of malignant tumours [37]

	Grade	Stage	Lymph node status	No of matched patient samples	No of WIF1 down-regulated samples	%
Urinary bladder						
Urothelial carcinoma	1–3	pTa–pT2	pN0	46	12	26.1
Prostate						
Adenocarcinoma	1–3	pT2a–pT4a	pN0–pN2	48	11	22.9
Breast						
Invasive ductal carcinoma	1–3	pT1b–pT4	pN0–pN2	27	17	63.0
Invasive lobular, mucinous, tubular	1–2	pT1b–pT4	pN0–pN1	8	4	50.0
Breast total				35	21	60.0
Lung						
Adenocarcinoma	1–3	pT1–pT3	pN0–pN2	11	4	36.4
Squamous cell carcinoma	1–3	pT1–pT4	pN0–pN2	22	21	95.5
Lung total				33	25	75.8

the Poly-A-tract 1000 kit (Promega, Heidelberg, Germany) according to the manufacturer's recommendations. After priming with the Affymetrix T7-oligo-dT promoter-primer combination (5′-GGCCAGTGAATT GTAATACGACTCACTATAGGGAGGCGGT$_{24}$- 3′ at 100 mM), first- and second-strand synthesis, and *in vitro* transcription, the amplified RNA (aRNA) was again amplified in two subsequent rounds of cDNA synthesis and *in vitro* transcription [26]. The cDNA from each round of amplification was tested for integrity by TaqMan PCR with the GAPDH assay (see below). Biotin-labelled nucleotides were incorporated into the aRNA in the last *in vitro* transcription. Hybridization and detection of the labelled aRNA were performed on the metg001A Affymetrix GeneChip according to the manufacturer's instructions.

Special algorithms were developed for data analysis. A non-parametric Wilcoxon test was used to test the probe sets for the presence or absence of expression signal. For significantly expressed probe sets, the 75% percentile of the perfect matches was used to calculate expression values after background subtraction and normalization. If an expression signal was not detected, the expression value was set to 0.1.

Quantitative RT-PCR

aRNA of one amplification cycle, as stated before, was reverse-transcribed to cDNA. The cDNA generated from 1 ng of aRNA was used for a Taqman assay (Applied Biosystems, Weiterstadt, Germany). The genes were amplified with the TaqMan Universal PCR Master Mix according to the manufacturer's conditions, using the ABI PRISM 5700 Sequence Detection System. The following oligonucleotides were used for real-time RT-PCR: WIF1 forward 5′-TAAAAGGTACGAAGCCAGCCTC-3′; WIF1 reverse 5′-GCGTGTGCTGCCTGAGC-3′; WIF1 probe 5′-FAM-CCTGAGGCCAGCAGGCGCC-Tamra-3′; GAPDH forward 5′-GAAGGTGAAGGTCGGAG TC-3′; GAPDH reverse 5′-GAAGATGGTGATGGGA TTTC-3′; GAPDH probe 5′-FAM-CAAGCTTCCCGT TCTCAGCC-Tamra-3′. cDNAs were quantified by the comparative C_T method, normalizing C_T values to GAPDH and calculating the relative expression values of normal to cancer tissue [27].

Northern blot hybridization

Multiple tissue northern blots and matched tumour/normal expression arrays were obtained from Clontech (Heidelberg, Germany). The matched tumour/normal expression array contains 68 tumour and normal samples from different patients as matched pairs from 12 different tissues and additional cell lines. Hybridizations with the α^{32}P-labelled DNA probe were performed according to the manufacturer's recommendations. The WIF1 probe was derived from a PCR reaction under standard conditions using 5 ng of mammary cDNA (Clontech, Heidelberg, Germany), 10 mM dNTPs, 0.5 mM primer, 1.5 mM $MgCl_2$, 10× reaction buffer, and 0.625 U of AmpliTaq Gold (Applied Biosystems, Weiterstadt, Germany), using the following primers: WIF1 forward 5′-GCATGGCCCGGAGGA-3′; WIF1 reverse 5′-TTTCATAAGATAGCATGTGCAAAG-3′. The reaction was performed in 35 cycles with an initial denaturation step of 10 min at 95 °C and then 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min. The identity of the PCR product was confirmed by sequencing. The arrays were hybridized according to the manufacturer's instructions and analysed using the Phosphoimager 595 (Molecular Dynamics, Sunnyvale, USA).

Immunohistochemistry and statistical analysis

Four-micrometre sections were cut from formalin-fixed and paraffin wax-embedded tissues and stained with a human polyclonal anti-WIF1 antibody directed to the WIF domain. Affinity-purified rabbit polyclonal anti-human WIF1 antibody was prepared as previously described [24]. In brief, rabbit anti-WIF-domain antibodies were raised against a fusion protein composed of the T7 gene 10 protein fused to amino acids 29–168 of human WIF1 and affinity-purified using an *E coli* maltose-binding protein fused to the same WIF1 fragment. After deparaffinization for 10 min in fresh Xylol, tissue sections were rehydrated in a series of diluted ethanol. For antigen retrieval, the slides were boiled for 10 min in a microwave oven in citrate buffer (pH 7.1). After incubation with the primary anti-WIF1 antibody at a 1:50 dilution, the ChemMate Kit (DAKO Diagnostics GmbH, Germany) was used, according to the manufacturer's protocol, to visualize the antibody–antigen reactivity. The provided chromogen DAB was applied in a 1:50 dilution. Finally, the slides were briefly counterstained with haematoxylin and eosin.

Immunohistochemical WIF1 expression was independently analysed by two pathologists (AH, MW). The scoring was based on the cytoplasmic perinuclear staining in tumour cells, compared with the corresponding normal epithelium. Nuclear and membrane staining was considered to be negative (0). Weak perinuclear cytoplasmic staining was defined as 1+, intermediate staining in less than 70% of the tumour cell population as 2+, and strong staining in more than 70% of the tumour cell population as 3+. Down-regulation of WIF1 protein was considered if the immunohistochemical staining intensity in the tumour was lower than in the corresponding normal epithelium. Equal staining intensities of tumour and normal tissues were defined as not dysregulated.

Differences were considered statistically significant when $p < 0.05$. A statistical correlation between clinicopathological and molecular parameters was tested using a two-sided Fisher exact test.

Results

Expression analysis of members of the Wnt pathway in prostate cancer

To provide high-quality starting material for our chip-based RNA expression analysis, we microdissected 54 prostate tumour samples. In prostate cancer, microdissection is absolutely required to reduce tumour heterogeneity and enrich tumour cells up to 90%. Similarly, matched normal epithelial cells were obtained from each of the 54 patients by microdissection. In order to study RNA profiles of the Wnt pathway, we extracted the expression values from the data set of our custom array (Table 1).

We defined the following criteria for differential gene expression: (i) the gene must be present in at least 27 (50%) of all prostate tumour patients; (ii) it must be up- or down-regulated in at least five (10%) tumour samples; and (iii) the degree of up- or down-regulation of the same gene should be at least two-fold.

Eleven genes fulfilled all three criteria. Of these, six genes including SFRP4, FZD4, FZD6, DVL1, TCF4, and MYC were up-regulated in prostate cancer patients. Secreted frizzled related protein 4 (SFRP4) was expressed in 37 of 54 (69%) patients and up-regulated in 30 (81%) of the SFRP4-expressing tumours. Of the five genes (WNT2, WIF1, PPP2CB, CCND2, CD44) down-regulated in prostate tumours, WIF1 was expressed in 42 of all 54 (78%) tumour samples. WIF1 was down-regulated in 27 of 42 (64%) WIF1-expressing tumours.

We also observed a wide range of RNA expression among members of the same protein family. For example, while the Wnt ligands WNT2 and WNT2b were expressed at an 80% level in all prostate cancer patients, WNT7a, WNT8b, and WNT14 were not expressed at all.

WIF1 is down-regulated in more than half of all prostate cancer patients

Our chip-based expression profiling experiments showed that WIF1 was down-regulated in 64% of the WIF1-expressing prostate tumour patients (Figure 1A). A more detailed examination of the data shows that WIF1 was completely lost at the RNA level in 18 tumours and significantly down-regulated in a further nine tumours. There was no correlation between complete loss and reduction of WIF1 at the RNA level with histopathological and clinical parameters including Gleason grade, tumour stage, and PSA values. Using quantitative PCR, we confirmed the down-regulation of WIF1 in 9 of 14 (64%) matched pairs (tumour/normal) of microdissected prostate tissue samples analysed (Figure 1B).

We then tested the differential expression of WIF1 in various types of human tumour by dot blot analysis using a commercial matched tumour/normal dot blot expression array containing samples from 68 individual patients with 11 different tumour entities. As shown in Figure 2, WIF1 was completely lost in all nine spotted breast tumours in comparison to the corresponding matched normal tissues. A similar result was observed for the lung cancer samples. They demonstrated a very weak WIF1 signal in all three cases and strong expression in the corresponding normal lung tissues.

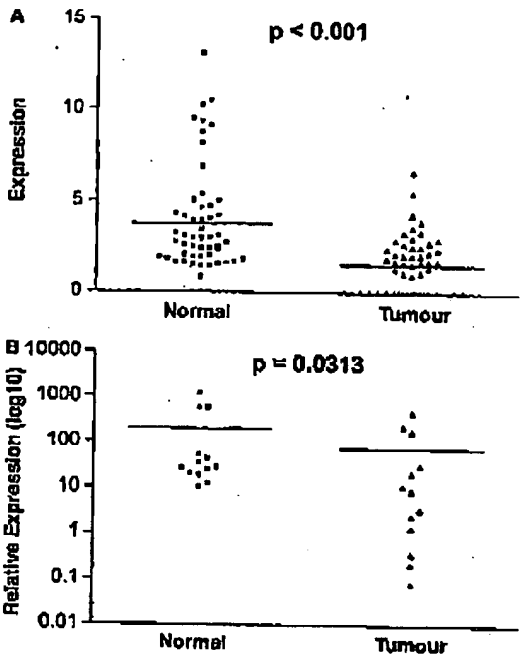

Figure 1. Differential RNA expression of WIF1 in prostate cancer by chip (A) and TaqMan (B) analysis. Expression values are defined as normalized arbitrary fluorescence units. Significance was calculated using the Mann–Whitney test

Distribution of WIF1 in normal tissues

The WIF1 gene has 10 exons and spans more than 200 kb on chromosome 12q14.3. The cDNA sequence as deposited in GenBank has a length of 2014 bp (NM_007191). However, this cDNA is still incomplete. In mice, only a single transcript could be identified [24]. We confirmed that the main human WIF1 transcript has a size of 2034 bp, as a 2.2 kb band was identified as the major message. In addition, three further WIF1-specific transcripts with lengths of 2.4, 2.7, and 3.2 kb were identified (Figure 3). The main 2.2 kb WIF1 message is highly abundant in human prostate, lung, and brain. In these tissues, it is expressed together with two less abundant isoforms of 2.4 and 3.2 kb. In skeletal muscle, we observed the 2.4 kb isoform as the only transcript.

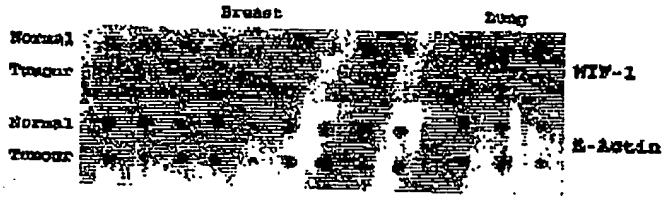

Figure 2. Hybridization of WIF1 and β-actin on the 'matched tumour/normal expression array' with cDNAs from different tissues and patients. Upper dots represent cDNA from normal; lower dots from tumour tissue. Only the samples of breast and lung tissue with a positive WIF1 signal are shown

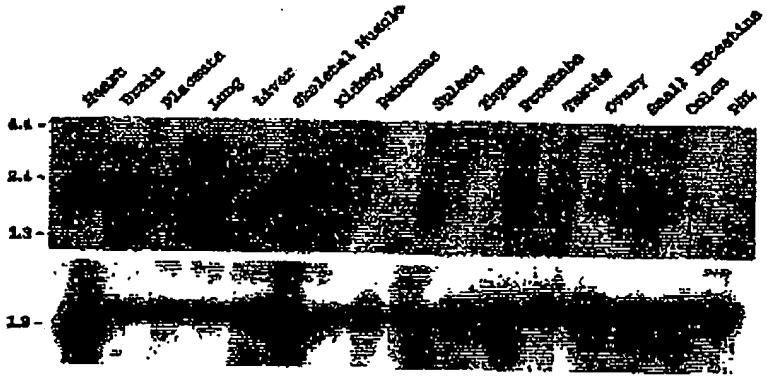

Figure 3. Multiple tissue northern (MTN)-northern blot analysis of WIF1 expression in 16 different human tissues

Immunohistochemistry of WIF1

In order to confirm the observed loss of RNA expression, a polyclonal WIF1 antibody [24] was used to test the protein expression of WIF1 in series of prostate, breast, lung, and bladder cancers.

The antibody showed a perinuclear cytoplasmic staining pattern as expected for a secreted protein and occasionally weak nuclear staining was found. However, no nuclear signal was detected in any of the tumours with weak or absent cytoplasmic staining. Membranous staining was not found in any tumour. WIF1 showed strong staining in endothelial cells in both small capillaries and larger vessels, whereas fibroblasts remained consistently negative. There was no evidence of differences in the staining intensities in endothelium of tumour vessels compared with vessels from normal tissue. There was strong staining of the normal epithelium in all the organs investigated.

Eleven of the 48 (23%) prostate cancers showed reduced WIF1 expression in comparison to the matching normal tissue. Interestingly, three patients demonstrated retention of WIF1 expression only in the well-differentiated tumour glands, but not in the poorly differentiated tumour areas (Gleason 4–5) (Figures 4A and 4B).

In 21/35 (60%) of the breast cancer specimens investigated, the expression of WIF1 was reduced in comparison with corresponding normal tissue (Figures 4C and 4D). In NSCLC, WIF1 was down-regulated at the protein level in 25 of the 33 (76%) cases investigated (Figures 4E and 4F).

WIF1 expression did not correlate with stage, grade or lymph node status in prostate, NSCLC, and breast cancer. Interestingly, however, reduction of WIF1 expression was significantly ($p = 0.001$) more frequent in squamous cell carcinomas (21/22, 96%) than in adenocarcinomas of the lung (4/11, 36%).

In bladder cancer, we observed loss of WIF1 expression in 12 of 46 (26%) cases investigated (Figures 4G and 4H). In invasive tumours (pT1–pT4), WIF1 loss was observed more frequently when compared with superficial papillary (pTa) tumours (pTa versus pT1–pT4; $p = 0.038$).

Discussion

A general problem of chip-based RNA expression analysis is the uncritical use of bulk tumour and 'normal' tissue as a source for RNA. It is well known that solid tumours are very heterogeneous and consist of several distinct cell types. In bulk tumour samples provided by surgeons or pathologists, the fraction of tumour cells is small and often below 30% of the entire cell mass. In prostate cancer, one can distinguish different tumour foci exhibiting different differentiation and grades within the same patient. In our view, microdissection is a prerequisite

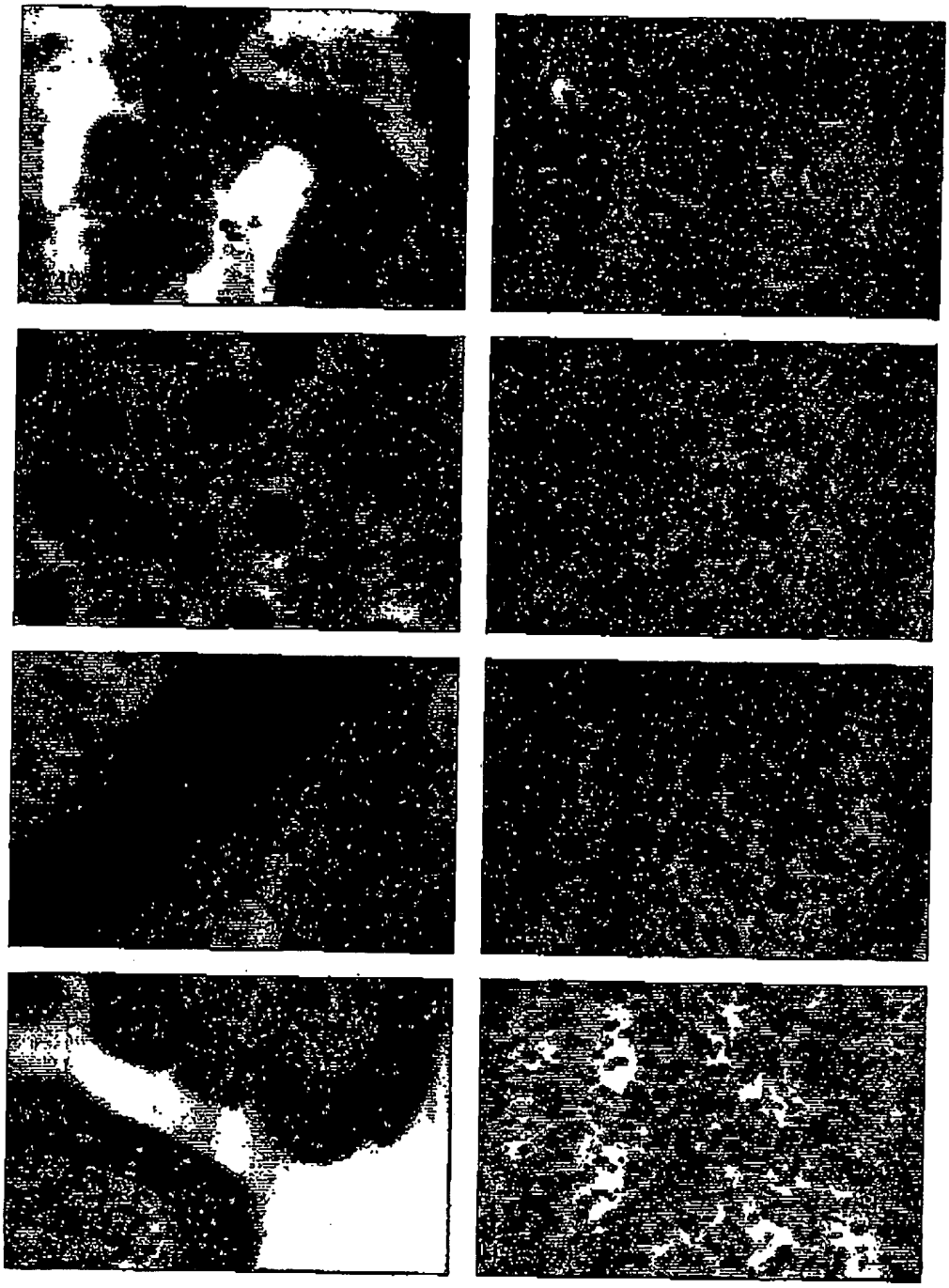

Figure 4. WIF1 immunohistochemistry. Normal prostate (A), breast (C), bronchial (E), and urinary bladder (G) tissue demonstrating positive perinuclear cytoplasmic WIF1 staining. There was a strong reduction or absence of WIF1 protein expression in the corresponding malignant tumours: prostate carcinoma (B), invasive ductal breast carcinoma (D), squamous cell lung carcinoma (F), and invasive urinary bladder carcinoma (H)

to the investigation of pure cell populations with defined histopathological characteristics in array-based experiments.

Members of the Wnt pathway have been implicated in a number of processes such as embryogenesis and carcinogenesis [28]. By regulation of different Wnt pathway components with antagonizing functions, the Wnt pathway is probably able to regulate tumour-promoting and -suppressing events. In order to identify transcriptionally regulated members of the Wnt pathway, we analysed the expression profiles of 54 microdissected prostate cancer and normal tissue samples by microarray analysis. In this study, we focused on 40 members of the Wnt pathway using a custom-made Affymetrix oligonucleotide chip.

We defined specific criteria for differential gene expression and applied them to our data set. Using these criteria, 11 genes were differentially expressed at the RNA level in microdissected matched prostate tumour/normal tissue samples. SFRP4, FZD4, FZD6, DVL1, TCF4, and MYC were found to be up-regulated, while WNT2, WIF1, PPP2CB, CCND2, and CD44 were down-regulated in prostate tumours.

The remaining 29 Wnt-pathway genes investigated were either not expressed in prostate or did not fulfil our criteria for differential expression. This might be due to different specificities in the binding and function of homologous genes of one gene family and differential expression of these genes in various tissue entities as shown in ref29. Despite their central role in the Wnt pathway, the key players β-catenin and APC were not regulated at the transcriptional level. The differential expression of extracellular components of the Wnt pathway is well known, particularly in bladder tumours [30], and involvement of intracellular factors such as APC or β-catenin was not observed [31].

From the genes encoding extracellular ligands of the Wnt pathway, we found for the first time that sFRP4 is up-regulated in prostate tumours. Interestingly, overexpression of sFRP4 has already been shown for endometrial carcinomas and invasive breast carcinomas [32]. Using immunohistochemistry, we further demonstrated down-regulation of WIF1 in prostate, breast, lung, and bladder cancers.

Recently, several groups have analysed the gene expression profiles obtained from bulk tissues including cancers of the prostate [1,33], breast [2,34], lung [3,35], and bladder [4]. They also made available their data sets for meta-analysis of single genes. WIF1 probes were not included in all experiments, but down-regulation of WIF1 was reported in prostate and lung carcinomas [1,35]. The expression profiles in lung cancers were similar to our immunohistochemistry results. However, reduced WIF1 protein expression was mainly observed in squamous cell carcinomas. Adenocarcinomas and squamous cell carcinomas, both NSCLCs, are preferentially located in different parts of the lung, differ in their RNA expression profile, and have a number of specific genetic alterations [3,35,36]. The observed down-regulation of the WIF1 protein may demonstrate a different regulation of the Wnt pathway in squamous cell carcinomas compared with adenocarcinomas.

Down-regulation of WIF1 was a common event in the tumours investigated. Down-regulation of the WIF1 protein correlated neither with tumour stage nor with grade or lymph node status of the prostate, breast, and NSCLC samples analysed. The absence of a correlation between the pathological features of the tumours and the loss of WIF1 expression strongly suggests that this could be an early carcinogenic event, at least in these tumour entities. In contrast to these results, WIF1 expression was more often lost in higher-stage bladder tumours, indicating a potential role in tumour progression.

Because WIF1 expression was reduced in a fraction of the tumour samples and varied between different tissues, there might be additional regulatory mechanisms for Wnt-pathway signal transduction, probably mediated by other extracellular ligands.

Recent studies have shown that the transcription of genes of the SFRP family is tightly regulated by promoter hypermethylation [23]. Suzuki *et al* observed hypermethylation of the promoters of SFRP1, SFRP2, SFRP4, and SFRP5 in human colon cell lines and primary colon cancers.

Our study demonstrates that chip expression profiling of microdissected primary cancer samples can be successfully used for the identification of transcriptionally regulated members of signal transduction pathways. Although some genes of the pathway have previously been identified as up- or down-regulated in cancer tissues, we have identified WIF1 as a new potential modulator of Wnt-pathway activity in prostate, breast, lung, and bladder cancer.

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